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(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US]; 9800 Medical Center Drive, Rockville, MD 20850 (US).			
(72) Inventors: RASHTCHIAN, Ayoub; 22418 Sweetleaf Lane, Gaithersburg, MD 20882 (US). SOLUS, Joseph; 4 Ivyberry Court, Gaithersburg, MD 20879 (US).			
(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).			
(54) Title: STABLE COMPOSITIONS FOR NUCLEIC ACID AMPLIFICATION AND SEQUENCING			
(57) Abstract			
<p>This invention is directed to compositions comprising mixtures of reagents, including thermostable enzymes (e.g., thermostable DNA polymerases), buffers, cofactors and other components, suitable for immediate use in nucleic acid amplification or sequencing techniques without dilution or addition of further components. The compositions contain no stabilizing agents (e.g., glycerol or serum albumin) and unexpectedly maintain activity for extended periods of time upon storage at temperature above freezing. These compositions are useful, alone or in the form of kits, for nucleic acid amplification (e.g., by the Polymerase Chain Reaction, as exemplified in the Figure) and sequencing (e.g., by dideoxy or "Sanger" sequencing), or for any procedure utilizing thermostable DNA polymerases in a variety of medical, forensic and agricultural applications. In particular, the compositions and methods are useful for amplifying and sequencing nucleic acid molecules that are larger than about 7 kilobases in size.</p>			

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Stable Compositions for Nucleic Acid Amplification and Sequencing

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FIELD OF THE INVENTION

This invention is in the fields of molecular and cellular biology. The invention is particularly directed to reagent compositions for use in techniques whereby nucleic acids (DNA or RNA) are amplified or sequenced, and to methods of amplifying and sequencing long nucleic acid molecules, especially via the polymerase chain reaction (PCR).

BACKGROUND OF THE INVENTION

15 DNA Polymerases

During growth and reproduction of viruses and cellular organisms, the DNA containing the parental genetic information must be faithfully copied and passed on to the progeny. This highly regulated process of DNA replication is carried out *in vivo* by a complex of enzymes and associated proteins and cofactors (Kornberg, A., *Science* 131:1503-1508, 1959; Hibner, U., and Alberts, B.M., *Nature* 285:300-305, 1980). The primary enzymes taking part in this process are the DNA polymerases, which catalyze the addition of deoxynucleoside triphosphate (dNTP) bases into the newly forming DNA strands. Together with other enzymes (*e.g.*, helicases, ligases and ATPases), the DNA polymerases thus ensure rapid and relatively faithful replication of DNA in preparation for proliferation in prokaryotes, eukaryotes and viruses.

25 DNA polymerases are also used to manipulate DNA *in vitro* in a variety of molecular genetic techniques. These enzymes have proven useful not only for *in vitro* DNA synthesis, but also for determining the nucleotide sequence (*i.e.*, "sequencing") of DNA fragments or genes. This latter application relies on the fact that, in addition to an activity which adds dNTPs to DNA in the 5' to 3' direction (*i.e.*, "polymerase" activity), many DNA polymerases also possess activities which
30 remove dNTPs in the 5' to 3' and/or the 3' to 5' direction (*i.e.*, "exonuclease" activity). DNA polymerases can also be used for sequencing via their incorporation of labelled chain-terminating agents such as dideoxynucleoside triphosphates (See U.S. Patent Nos. 4,962,020; 5,173,411; and 5,498,523). Thus the same enzyme, *e.g.*, DNA polymerases I and III from the bacterium *Escherichia coli*, DNA polymerase γ from animal cells or DNA polymerase from bacteriophage T7 (See U.S.
35 Patent No. 4,795,699), may be used *in vitro* both for DNA synthesis, involving the elongation of the

DNA strand, and for DNA sequencing, involving either the synthesis or the digestion of the DNA strand.

The dual activity of certain DNA polymerases is, however, a drawback for some *in vitro* applications. For example, the *in vitro* synthesis of an intact copy of a DNA fragment by the polymerase activity, an elongation process which proceeds in a 5' to 3' direction along the template DNA strand, is jeopardized by the exonuclease activities which may simultaneously or subsequently degrade the newly formed DNA. To overcome this technical problem, a fragment of *E. coli* DNA polymerase I lacking the 5' to 3' exonuclease activity (named the "Klenow fragment" after its discoverer) is often employed for *in vitro* DNA synthesis. The Klenow fragment provides for *in vitro* DNA synthesis at approximately the same rate as intact *E. coli* DNA polymerase I, but the newly synthesized DNA molecules are less subject to enzymatic degradation and are correspondingly more stable.

Unfortunately, the error rate (*i.e.*, the rate at which incorrect dNTPs are incorporated into the new DNA strand by the enzyme) is somewhat higher for the Klenow fragment than for many other commonly used DNA polymerases, including *E. coli* DNA polymerases I and II and the polymerases from bacteriophages T4 and T7 (Kunkel, T.A., *et al.*, *J. Biol. Chem.* 259:1539-1545, 1984; Tindall, K.R., and Kunkel, T.A., *Biochemistry* 27:6008-6013, 1988; Mattila, P., *et al.*, *Nucl. Acids Res.* 19:4967-4973, 1991). Thus, until recently the rates of synthesis, degradation and error had to be weighed together when choosing which DNA polymerase to use for *in vitro* DNA synthesis.

DNA Sequencing

In general, two techniques have been traditionally used to sequence nucleic acids. In the first method, termed "Maxam and Gilbert sequencing" after its co-developers (Maxam, A.M. and Gilbert, W., *Proc. Natl. Acad. Sci. USA* 74:560-564, 1977), DNA is radiolabeled, divided into four samples and treated with chemicals that selectively destroy specific nucleotide bases in the DNA and cleave the molecule at the sites of damage. By separating the resultant fragments into discrete bands by gel electrophoresis and exposing the gel to X-ray film, the sequence of the original DNA molecule can be read from the film. This technique has been used to determine the sequences of certain complex DNA molecules, including the primate virus SV40 (Fiers, W., *et al.*, *Nature* 273:113-120, 1978; Reddy, V.B., *et al.*, *Science* 200:494-502, 1978) and the bacterial plasmid pBR322 (Sutcliffe, G., *Cold Spring Harbor Symp. Quant. Biol.* 43:77-90, 1979).

An alternative technique for sequencing, named "Sanger sequencing" after its developer (Sanger, F., and Coulson, A.R., *J. Mol. Biol.* 94:444-448, 1975), is more commonly employed. This method uses the DNA-synthesizing activity of DNA polymerases which, when combined with mixtures of reaction-terminating dideoxynucleoside triphosphates (Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA* 74:5463-5467, 1977) and a short primer (either of which may be detectably labeled), gives rise to a series of newly synthesized DNA fragments specifically terminated at one of the four dideoxy bases. These fragments are then resolved by gel electrophoresis and the sequence determined as described for Maxam and Gilbert sequencing above. By carrying out four separate reactions (once with each ddNTP), the sequences of even fairly complex DNA molecules may rapidly be determined (Sanger, F., *et al.*, *Nature* 265:678-695, 1977; Barnes, W., *Meth. Enzymol.* 152:538-556, 1987). While Sanger sequencing usually employs *E. coli* or T7 DNA polymerase (U.S. Patent No. 4,795,699), recent modifications of this technique using T7 polymerase mutants allow sequencing to be accomplished using a single sequencing reaction containing all four chain-terminating ddNTPs at different concentrations (U.S. Patent Nos. 4,962,020 and 5,173,411). Further modifications to the technique, to reduce or eliminate the buildup of reaction-poisoning pyrophosphate in the reaction mixtures, have also been described (U.S. Patent No. 5,498,523).

The Polymerase Chain Reaction

Soon after their identification and characterization, it was recognized that the activities of the various enzymes and cofactors involved in DNA synthesis could be exploited *in vitro* to dramatically increase the concentration of, or "amplify," one or more selected DNA sequences. For many medical, diagnostic and forensic applications, amplification of a particular DNA sequence is essential to allow its detection in, or isolation from, a sample in which it is present in very low amounts. More recently, *in vitro* amplification of specific genes has provided powerful and less costly means to facilitate the production of therapeutic proteins by molecular biological techniques, and may have applications in genetic therapy as well.

While a variety of nucleic acid amplification processes has been described, the most commonly employed is the Polymerase Chain Reaction (PCR) technique disclosed in U.S. Patent Nos. 4,683,195 and 4,683,202. In this process, a sample containing the nucleic acid sequence to be amplified (the "target sequence") is first heated to denature or separate the two strands of the nucleic acid. The sample is then cooled and mixed with specific oligonucleotide primers which hybridize to the target sequence. Following this hybridization, DNA polymerase in a buffered aqueous solution

is added to the sample, along with a mixture of the dNTPs that are linked by the polymerase to the replicating nucleic acid strand. After allowing polymerization to proceed to completion, the products are again heat-denatured, subjected to another round of primer hybridization and polymerase replication, and this process repeated any number of times. Since each nucleic acid product of a given cycle of this process serves as a template for production of two new nucleic acid molecules (one from each parent strand), the PCR process results in an exponential increase in the concentration of the target sequence. Thus, in a well-controlled, high-fidelity PCR process, as few as 20 cycles can result in an over one million-fold amplification of the target nucleic acid sequence (See U.S. Patent Nos. 4,683,195 and 4,683,202).

Thermostable DNA Polymerases

Overview

Initially, the DNA polymerases of choice for use in DNA sequencing or PCR were *E. coli* DNA polymerase I, the Klenow fragment, or T4 or T7 polymerases owing to their ease of isolation and well-characterized activities. However, the use of these enzymes necessitated their addition prior to the start of each sequencing or PCR cycle, due to their thermolability at the temperatures used to denature the DNA strands in the initial steps of the processes (typically 70° to 95°C) (Saiki, R.K., *et al.*, *Science* 230:1350-1354, 1985; Mullis, K.B., and Faloona, F.A., *Meth. Enzymol.* 155:335-350, 1987; U.S. Patent No. 4,795,699). This need for the addition of fresh enzyme at the beginning of each cycle increased the amount of time required for these processes, and increased the risk of operator error and contamination of the samples during reagent introduction, often leading to undesirable results.

These difficulties were partially overcome by the use of thermostable DNA polymerases in the PCR process (Saiki, R.K., *et al.*, *Science* 239:487-491, 1988). The thermostable DNA polymerase most commonly used in PCR is *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus* (Saiki *et al.*, 1988, *Id.*; U.S. Patent Nos. 4,889,818 and 4,965,188). *Taq* polymerase functions optimally at temperatures of 70-80°C, and is able to maintain substantial activity upon repeated exposure to temperatures of 92°-95°C as are often used in the initial steps of PCR (Gelfand, D.H., and White, T.J., *in: PCR Protocols: A Guide to Methods and Applications*, Innis, M.A., *et al.*, eds., Academic Press, pp. 129-141, 1989; Bej, A.K., and Mahbubani, M.H., *in: PCR Technology: Current Innovations*, Griffin, H.G., and Griffin, A.M., eds., CRC Press, pp. 219-237, 1994).

The use of *Taq* polymerase in PCR eliminated the need to add fresh enzyme to the reaction mix prior to each PCR cycle. Instead, a quantity of *Taq* polymerase sufficient to catalyze DNA polymerization over the desired number of cycles can be mixed with the other components prior to the initiation of the first PCR cycle, and the enzyme continues to function throughout the repetitive cycles of increased and decreased temperatures. The use of *Taq* polymerase has also facilitated the automation of the PCR process (Gelfand and White, *Id.*), thereby at once dramatically reducing time constraints and the risks of operator error and sample contamination that are problematic with thermolabile polymerases. Currently, most PCR amplification of nucleic acids for industrial and academic applications is performed using *Taq* polymerase and automated thermal cycling instrumentation.

In addition to *Taq* polymerase, other thermostable polymerases have found similar application in PCR (Bej and Mahbubani, *Id.*). Particularly useful as substitutes for *Taq* polymerase in PCR are polymerases isolated from the thermophilic bacteria *Thermus thermophilus* (*Tth* polymerase), *Thermococcus litoralis* (*Tli* or VENTTM polymerase), *Pyrococcus furiosus* (*Pfu* or DEEPVENT polymerase), *Pyrococcus woosii* (*Pwo* polymerase) and other *Pyrococcus* species, *Bacillus sterothermophilus* (*Bst* polymerase), *Sulfolobus acidocaldarius* (*Sac* polymerase), *Thermoplasma acidophilum* (*Tac* polymerase), *Thermus flavus* (*Tfl/Tub* polymerase), *Thermus ruber* (*Tru* polymerase), *Thermus brockianus* (DYNAZYMETM polymerase), *Thermotoga neapolitana* (*Tne* polymerase; See WO 96/10640), *Thermotoga maritima* (*Tma* polymerase; See U. S. Patent No. 5,374,553) and other species of the *Thermotoga* genus (*Tsp* polymerase) and *Methanobacterium thermoautotrophicum* (*Mth* polymerase). While each of these polymerases is useful for particular applications (See Bej and Mahbubani, *Id.*, p. 222), *Taq* polymerase is still by far the most commonly used polymerase in PCR.

Thermostable polymerases have also found application in DNA sequencing techniques, particularly in automated methods of dideoxy sequencing such as "cycle sequencing." These approaches resemble PCR in most respects except that, in place of dNTPs, automated DNA sequencing uses ddNTPs which allow determination of the sequence of the template DNA as described above. Use of higher denaturation temperatures in automated sequencing also improves sequencing efficiency (*i.e.*, fewer misincorporations occur) and allows the sequencing of templates that are GC-rich or contain significant secondary structure (such as supercoiling).

The use of thermolabile DNA polymerases such as *E. coli* or T7 DNA polymerases in these approaches, however, is subject to the same limitations described above for their use in PCR.

Accordingly, automated methods of DNA sequencing utilizing higher temperatures have increasingly employed thermostable DNA polymerases, the most commonly used of which is, as for PCR, *Taq* polymerase.

5 *Technical Limitations*

The use of *Taq* and other thermostable polymerases in sequencing and PCR is not, however, without drawback. For example, the error rate for *Taq* polymerase is substantially higher (*i.e.*, the final product is of "lower fidelity") than that for most of the thermolabile DNA polymerases, including the Klenow fragment of *E. coli* DNA polymerase I (Tindall and Kunkel, *Id.*), averaging
10 about 10^4 misincorporations per base pair per cycle. In addition, *Taq* polymerase is only useful for amplifying relatively short stretches of DNA (maximum length on the order of 5-6 kilobases; Barnes, W.M., *Proc. Natl. Acad. Sci. USA* 91:2216-2220, 1994), thus precluding its use in PCR amplification of large genes and whole genomes as is necessary in many current applications.

These technical limitations are apparently related: it has been theorized that the *Taq*
15 polymerase PCR length limitation is due to the low efficiency of elongation of the newly synthesized DNA strands at the sites of incorporation of mismatched bases in the parent strands (Barnes, *Id.*). Further contributing to this difficulty is the absence in *Taq* polymerase of a 3' to 5' exonuclease activity, which in other polymerases acts in a "proofreading" capacity to correct these mismatches and reduce the error rate (Bej and Mahbubani, *Id.*). The 5' to 3' exonuclease activity present in most
20 thermostable DNA polymerases can also degrade the 5' ends of the oligonucleotide primers (also a complication with the 3' to 5' exonuclease activity which can degrade the 3' ends of the primers), yielding undesirable results due to an early termination of the PCR process (*See* WO 92/06200; Barnes, *Id.*).

In sequencing reactions, *Taq* polymerase is subject to a limitation shared by *E. coli*
25 polymerase I and the Klenow fragment. These enzymes each are "discriminatory," meaning that they preferentially incorporate dNTPs over ddNTPs into newly synthesized DNA. Thus, to use *Taq* polymerase in automated sequencing reactions, relatively high concentrations of ddNTPs must be maintained in the reaction mixtures, to kinetically favor ddNTP incorporation by the enzyme. This need for high levels of ddNTPs can be prohibitively expensive, particularly when large DNA
30 fragments are being sequenced.

As another technical limitation, the DNA polymerases have heretofore been maintained in highly concentrated stock solutions in storage buffers containing glycerol, bovine serum albumin

and/or other stabilizing agents and stored at -20°C or lower (See, e.g., WO 92/06188; U.S. Patent No. 5,436,149). The conventional understanding in the field has been that the enzymes would rapidly lose activity at more dilute working concentrations (as do many bioactive proteins) and in solutions without glycerol or other stabilizing agents. Moreover, the solutions of enzymes had to be mixed with dNTPs or ddNTPs, cofactors (such as Mg⁺⁺) and one or more detergents immediately prior to use in the sequencing or PCR processes, as it was believed that premixture and storage of these solutions would also deleteriously affect their stability. In addition, dNTPs have traditionally been stored at temperatures below -20°C and have also been thought to be unstable if stored otherwise (Maniatis, T., *et al.*, *Molecular Cloning, A Laboratory Manual*, CRC Press, 1992). Together, these limitations have made the use of compositions containing thermostable DNA polymerases in DNA sequencing and PCR more costly and time-consuming than would be desired.

Overcoming Technical Limitations

Several approaches have been undertaken to attempt to surmount these technical difficulties. The results of DNA sequencing methodologies, for example, have been improved by the use of mutant *Taq* enzymes such as ΔTaq (WO 92/06188) lacking the 5' to 3' exonuclease activity. Improved sequencing results have also been obtained by including in the reaction mixture an agent such as pyrophosphatase which breaks down the pyrophosphate that can be formed during dideoxy sequencing reactions (See U.S. Patent Number 5,498,523). To overcome the discrimination between ddNTPs and dNTPs, some investigators have used T7 DNA polymerase in sequencing, as this enzyme is "nondiscriminatory," meaning that it incorporates ddNTPs at approximately the same rate as dNTPs (See U.S. Patent No. 4,795,699). Alternatively, mutants of DNA polymerase from a variety of organisms (e.g., *E. coli*) which are nondiscriminatory have also been described; see copending U.S. Patent Application No. 08/525,057 of Deb K. Chatterjee, filed September 8, 1995, entitled "Mutant DNA Polymerases and Use Thereof," the disclosure of which is expressly incorporated herein by reference. However, as described above, both *E. coli* and T7 DNA polymerases are thermolabile, so their use in automated sequencing requires addition of fresh enzyme at the beginning of each cycle.

More recently, mutations in *The* polymerase from *Thermotoga neapolitana* have been described, which overcome these limitations (WO 96/10640). One of these mutations, in which a phenylalanine residue at amino acid position number 730 in the wildtype protein (SEQ ID NO:1) is replaced with a tyrosine residue, results in a mutant *The* polymerase (SEQ ID NO:2) which is both

thermostable *and* nondiscriminatory. This mutant *Tne* polymerase thus provides a solution to both the problems of thermolability and ddNTP discrimination found in other enzymes used in automated DNA sequencing. See also the co-pending U.S. Patent Application of A. John Hughes and Deb K. Chatterjee, entitled "Cloned DNA Polymerases from *Thermotoga* and Mutants Thereof," filed on even
5 day herewith, which is incorporated by reference herein in its entirety.

In PCR applications, the low fidelity of *Taq*-produced PCR products has been alleviated to some extent by the use of *Pfu* DNA polymerase which contains the proofreading 3' to 5' exonuclease activity lacking in *Taq* polymerase (Lundberg, K.S., *et al.*, *Gene* 108:1-6, 1991). Other thermostable DNA polymerases, including *Tli*/VENT™ (Bej and Mahbubani, *Id.*) and DEEPVENT™ (Flaman,
10 J.-M., *et al.*, *Nucl. Acids Res.* 22(15):3259-3260, 1994) have also been shown to improve the fidelity of PCR products.

As a means of overcoming this length limitation, mutant enzymes lacking the 5' to 3' exonuclease activity have been prepared, including N-terminal deletion mutants of *Taq* polymerase that are analogous to the Klenow fragment of *E. coli* DNA polymerase I. Several of these mutant
15 enzymes, including Klentaq-1 and Klentaq-278 (Barnes, W.M., *Gene* 112:29-35, 1992; U.S. Patent No. 5,436,149), the *Taq* Stoffel fragment (Lawyer, F.C., *et al.*, *PCR Meth. Appl.* 2:275-287, 1993), and mutants of other thermostable DNA polymerases lacking the 5' to 3' exonuclease activity (*e.g.*, those disclosed in WO 92/06200), have been shown to provide increasingly stable PCR products and primers. However, as these enzymes also lack the 3' to 5' proofreading activity, their use subjects
20 the PCR process to the increased error rates described above. Thus, even with these mutant enzymes, high-fidelity PCR amplification of DNA fragments larger than 5-6 kilobases has proven exceedingly difficult.

By combining specific quantities of several enzymes, however, high-fidelity PCR amplification of large DNA sequences has been achieved. For example, use of a combination of a high
25 concentration of a thermostable DNA polymerase lacking the 3' to 5' exonuclease activity (*e.g.*, Klentaq278) and a low concentration of a thermostable DNA polymerase exhibiting the 3' to 5' exonuclease activity (*e.g.*, *Pfu*/DEEPVENT or *Tli*/VENT™) provides for amplification to high concentrations of DNA sequences of at least 35 kilobases in length with significantly improved fidelity (Barnes, *Id.*; U.S. Patent No. 5,436,149). Similar results have been obtained with mixtures
30 of *Tth* polymerase and low levels of thermostable polymerases from *Pyrococcus*, *Tli* or *Tma* (U.S. Patent No. 5,512,462). Apparently, the low level of 3' to 5' exonuclease activity is sufficient to remove any mismatched bases incorporated by the majority polymerase, but is insufficient to

significantly degrade the primers. While this approach has heretofore been applied to simple DNA sequences such as those from bacteriophage λ , it may prove applicable to larger and more complex sequences as well, including those of the genomes of bacteria, yeast, plants and animals.

Despite these successes in overcoming the ddNTP discrimination, length and fidelity
5 limitations, however, compositions comprising the reagents necessary for DNA sequencing or PCR capable of extended storage above freezing at working concentrations, without stabilizing agents, have not heretofore been reported. Thus, the time and economic constraints to the use of solutions of thermostable enzymes in most applications have yet to be overcome.

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BRIEF SUMMARY OF THE INVENTION

The present invention overcomes these temporal and economic limitations of previously available reagent compositions used in nucleic acid amplification and sequencing methods. Specifically, the invention is directed to compositions comprising mixtures of reagents at working
15 concentrations suitable for use with or without dilution and maintaining activity upon storage for an extended time, said mixtures consisting essentially of at least one thermostable enzyme and at least one buffer salt. The invention further provides such compositions for use in nucleic acid amplification further comprising at least one deoxynucleoside triphosphate, magnesium salts and at least one nonionic detergent, wherein the thermostable enzyme is at least one thermostable DNA
20 polymerase. In another embodiment, the invention provides such compositions for use in nucleic acid sequencing further comprising at least one deoxynucleoside triphosphate, at least one dideoxynucleoside triphosphate, magnesium salts and at least one nonionic detergent, wherein the thermostable enzyme is at least one thermostable DNA polymerase. More specifically, the invention is directed to such compositions wherein the thermostable DNA polymerase is *Taq*, *Tne*, *Tma*, *Pfu*,
25 *Pwo* or *Tth* DNA polymerase, or mutants thereof, preferably at concentrations of about 0.1-200 units per milliliter, about 0.1-50 units per milliliter, about 0.1-40 units per milliliter, about 0.1-36 units per milliliter, about 0.1-34 units per milliliter, about 0.1-32 units per milliliter, about 0.1-30 units per milliliter, or about 0.1-20 units per milliliter, and most preferably at concentrations of about 20 units per milliliter. The invention is also directed to such compositions that further comprise VENT or
30 DEEPVENT™ DNA polymerase, preferably at concentrations of about 0.0002-200 units per milliliter, about 0.002-100 units per milliliter, about 0.002-20 units per milliliter, about 0.002-2.0 units per milliliter, about 0.002-1.6 units per milliliter, about 0.002-0.8 units per milliliter, about

0.002-0.4 units per milliliter, or about 0.002-0.2 units per milliliter, most preferably at concentrations of about 0.40 units per milliliter. The invention is further directed to kits for DNA amplification or sequencing, said kits comprising a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles or the like, wherein a first container means contains a composition comprising a mixture of reagents at working concentrations suitable for use without dilution and maintaining activity upon storage for extended time, said mixture consisting essentially of at least one thermostable DNA polymerase, buffer salts, magnesium salts and at least one nonionic detergent. The first container means may also contain a mixture of dNTPs (for PCR applications) or ddNTPs (for sequencing applications).

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10 Alternatively, the dNTPs or ddNTPs may be included in a second container means also closely confined within the carrier means of the kit.

The invention is also directed to methods of amplifying or sequencing a nucleic acid molecule, comprising contacting the nucleic acid molecule to be amplified or sequenced, which is preferably larger than about 4-8 kilobases in size, more preferably larger than about 5-7 kilobases in size and most preferably larger than about 7 kilobases in size, with the compositions of the invention. The invention also provides nucleic acid molecules amplified by these methods.

The present invention is also directed more generally to compositions containing thermostable proteins or enzymes useful in molecular biology. These compositions comprise mixtures of reagents at working concentrations suitable for use with or without dilution which maintain the enzyme or protein activity upon storage for an extended time. The compositions of this aspect of the invention comprise at least one thermostable enzyme and at least one buffer salt. The thermostable enzymes in these compositions include, but are not limited to, polymerases, restriction enzymes, alkaline phosphatases, reverse transcriptases, ligases, nucleases, pyrophosphatases, DNAses, RNAses, exonucleases, RNase inhibitors, kinases, topoisomerases, guanylyltransferases and glycosylases (*e.g.*, uracil DNA glycosylase).

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30 The invention is further directed to kits for conducting a procedure associated with the thermostable enzymes or proteins (restriction enzymes, phosphatases, etc.), the kits comprising a container means such as a box, carton, tube and the like, having in close confinement therein one or more container means, such as a vial, tube, ampule, bottle or the like, wherein a first container means contains a composition comprising a mixture of reagents at working concentrations suitable for use with or without dilution. The reagents in the first container means include at least one thermostable enzyme or protein and at least one buffer salt.

The compositions of the invention have unexpectedly been found to maintain enzyme activity for extended periods of time compared to conventional compositions. For example, the present compositions maintain enzyme activity for at least four weeks when stored at ambient temperature (about 20°-25°C), for at least one year at about 4°C and for at least two years at about -20°C.

5 These stable, ready-to-use reagent compositions are useful for nucleic acid amplification (including the Polymerase Chain Reaction or PCR) and sequencing (including dideoxy sequencing), or for any procedure using thermostable DNA polymerases or other enzymes (restriction enzymes, phosphatases, kinases, etc.) in fields such as medical therapeutics and diagnostics, forensics and agricultural science.

10 Other features, advantages and applications of the present invention will be apparent to those skilled in the art from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

15 **Figure 1** is a photograph of an agarose gel (visualized by ethidium bromide fluorescence under ultraviolet illumination) of PCR amplification of a 1.3 kilobase human genomic DNA fragment from 10 nanograms of template using 15 different reagent compositions (corresponding to samples 10-24 in Table 1) stored as indicated in Table 1, and a freshly made control sample. Lane marked

20 "M" contains markers indicating amount of DNA loaded; bands correspond to (from top to bottom) 100 nanograms, 60 nanograms, 40 nanograms and 20 nanograms of DNA mass markers.

25 **Figure 2** is a photograph of an agarose gel (visualized by ethidium bromide fluorescence under ultraviolet illumination) of PCR amplification of a 4.1 kilobase human genomic DNA fragment using the amounts indicated (in nanograms) of template for each amplification reaction. The samples on the upper portion of the gel were amplified with a nucleic acid amplification composition stored for 10 weeks at 4°C, while those on the lower portion of the gel were amplified with a composition stored for 10 weeks at ambient temperature (about 20-25°C). Leftmost lanes in each portion are markers as indicated in Figure 1.

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DETAILED DESCRIPTION OF THE INVENTION

Definitions

Throughout this disclosure, various terms that are generally understood by those of routine skill in the art are used. Certain terms as used herein, however, have specific meanings for the purposes of the present invention. The term "dNTP" (plural "dNTPs") generically refers to the deoxynucleoside triphosphates (*e.g.*, dATP, dCTP, dGTP, dTTP, dUTP, dITP, 7-deaza-dGTP, α dATP, α dTTP, α dGTP and α dCTP), and the term "ddNTP" (plural "ddNTPs") to their dideoxy counterparts, that are incorporated by polymerase enzymes into newly synthesized nucleic acids. The term "unit" as used herein refers to the activity of an enzyme. When referring to a thermostable DNA polymerase, one unit of activity is the amount of enzyme that will incorporate 10 nanomoles of dNTPs into acid-insoluble material (*i.e.*, DNA or RNA) in 30 minutes under standard primed DNA synthesis conditions. "Working concentration" is used herein to mean the concentration of a reagent that is at or near the optimal concentration used in a solution to perform a particular function (such as amplification, sequencing or digestion of nucleic acids). The term "detergent" as used herein refers to a nonionic surfactant such as TRITON X-100®, Nonidet P-40 (NP-40), Tween 20 or Brij 35. The terms "stable" and "stability" as used herein generally mean the retention by an enzyme of at least 70%, preferably at least 80%, and most preferably at least 90%, of the original enzymatic activity (in units) after the enzyme or composition containing the enzyme has been stored for at least four weeks at a temperature of about 20-25°C, at least one year at a temperature of about 4°C or at least 2 years at a temperature of -20°C.

Overview

The present invention provides, in a first preferred embodiment, compositions comprising mixtures of at least one thermostable enzyme (*e.g.*, a thermostable DNA polymerase, restriction enzyme, etc.), at least one buffer salt, and other reagents necessary for carrying out the procedure associated with the enzyme(s) (*e.g.*, deoxynucleoside triphosphates (dNTPs) for amplification of nucleic acids, dNTPs and dideoxynucleoside triphosphates (ddNTPs) for sequencing of nucleic acids, etc.). These compositions contain no stabilizing compounds (*e.g.*, glycerol, serum albumin or gelatin) that have been traditionally included in stock reagent solutions, and exhibit increased stability (measured as maintenance of enzyme activity) even upon storage at temperatures above freezing. Furthermore, the invention provides these reagent compositions in ready-to-use concentrations,

obviating the time-consuming dilution and pre-mixing steps necessary with previously available solutions. Unexpectedly, even at these diluted concentrations the reagent compositions are stable for extended periods of time at temperatures ranging from ambient (about 20-25°C) to about -70°C.

In additional preferred embodiments, the present invention provides these ready-to-use compositions in the form of kits that are suitable for immediate use to carry out the procedure associated with the enzyme(s) (e.g., nucleic acid amplification or sequencing in the case of DNA polymerases). These kits are also stable for extended periods of time at temperatures ranging from ambient (about 20-25°C) to -70°C.

10 Sources of Reagents

The compositions of the present invention may be formed by mixing the component reagents at the concentrations described below. The components for making the ready-to-use compositions can be obtained from, for example, GIBCO/LTI (Gaithersburg, Maryland).

15 *Thermostable Enzymes*

The thermostable enzymes (e.g., DNA polymerases, restriction enzymes, phosphatases, etc.) used in the present invention may be isolated from natural or recombinant sources, by techniques that are well-known in the art (See Bej and Mahbubani, *Id.*; WO 92/06200; WO 96/10640), from a variety of thermophilic bacteria that are available commercially (for example, from American Type Culture Collection, Rockville, Maryland) or may be obtained by recombinant DNA techniques (WO 20 96/10640). Suitable for use as sources of thermostable enzymes or the genes thereof for expression in recombinant systems are the thermophilic bacteria *Thermus thermophilus*, *Thermococcus litoralis*, *Pyrococcus furiosus*, *Pyrococcus woosii* and other species of the *Pyrococcus* genus, *Bacillus sterothermophilus*, *Sulfolobus acidocaldarius*, *Thermoplasma acidophilum*, *Thermus flavus*, 25 *Thermus ruber*, *Thermus brockianus*, *Thermotoga neapolitana*, *Thermotoga maritima* and other species of the *Thermotoga* genus, and *Methanobacterium thermoautotrophicum*, and mutants thereof. It is to be understood, however, that thermostable enzymes from other organisms may also be used in the present invention without departing from the scope or preferred embodiments thereof. As an alternative to isolation, thermostable enzymes (e.g., DNA polymerases) are available 30 commercially from, for example, GIBCO/LTI (Gaithersburg, Maryland), New England Biolabs (Beverly, Massachusetts), Finnzymes Oy (Espoo, Finland) and Perkin Elmer Cetus (Norwalk,

Connecticut). Once obtained, the purified enzymes may be placed into solution at working concentrations and stored according to the methods of the present invention.

dNTPs

5 The dNTP components of the present compositions serve as the "building blocks" for newly synthesized nucleic acids, being incorporated therein by the action of the polymerases. These dNTPs -- deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), and for some applications deoxyuridine triphosphate (dUTP) and deoxyinosine triphosphate (dITP), α -thio-dATP and 7-deaza-dGTP -- are
10 available commercially from sources including GIBCO/LTI (Gaithersburg, Maryland), New England Biolabs (Beverly, Massachusetts) and Sigma Chemical Company (Saint Louis, Missouri). The dNTPs may be unlabeled, or they may be detectably labeled by coupling them by methods known in the art with radioisotopes (*e.g.*, ^3H , ^{14}C , ^{32}P or ^{35}S), vitamins (*e.g.*, biotin), fluorescent moieties (*e.g.*, fluorescein, rhodamine, Texas Red, or phycoerythrin) or other detection agents. Labeled dNTPs may
15 also be obtained commercially, for example from GIBCO/LTI (Gaithersburg, Maryland) or Sigma Chemical Company (Saint Louis, Missouri). Once obtained, the dNTPs may be placed into solution at working concentrations and stored according to the methods of the present invention.

ddNTPs

20 The ddNTP components of the present compositions serve as the "terminating agents" in the dideoxy nucleic acid sequencing methodologies, being incorporated into newly synthesized nucleic acids by the action of the polymerases. These ddNTPs -- dideoxyadenosine triphosphate (ddATP), dideoxycytosine triphosphate (ddCTP), dideoxyguanosine triphosphate (ddGTP), dideoxythymidine triphosphate (ddTTP), and for some applications dideoxyuridine triphosphate (ddUTP) and
25 dideoxyinosine triphosphate (ddITP) -- are available commercially from sources including GIBCO/LTI (Gaithersburg, Maryland), New England Biolabs (Beverly, Massachusetts) and Sigma Chemical Company (Saint Louis, Missouri). The ddNTPs may be unlabeled, or they may be detectably labeled by coupling them by methods known in the art with radioisotopes (*e.g.*, ^3H , ^{14}C , ^{32}P or ^{35}S), vitamins (*e.g.*, biotin), fluorescent moieties (*e.g.*, fluorescein, rhodamine, Texas Red, or
30 phycoerythrin) or other detection agents. Labeled ddNTPs may also be obtained commercially, for example from GIBCO/LTI (Gaithersburg, Maryland) or Sigma Chemical Company (Saint Louis,

Missouri). Once obtained, the ddNTPs may be placed into solution at working concentrations and stored according to the methods of the present invention.

Buffers/Salts

5 All buffers and cofactor salts comprising the compositions of the present invention, and concentrated stock solutions thereof, are available from a variety of commercial sources including GIBCO/LTI (Gaithersburg, Maryland) and Sigma Chemical Company (Saint Louis, Missouri). Particularly preferred buffers for use in forming the present compositions are the sulfate, hydrochloride, phosphate or free acid forms of tris-(hydroxymethyl)aminomethane (TRIS®),
10 although alternative buffers of the same approximate ionic strength and pKa as TRIS® may be used with equivalent results. In addition to the buffer salts, cofactor salts such as those of potassium (preferably potassium chloride) and magnesium (preferably magnesium chloride or sulfate) are included in the compositions. Once obtained, the buffers and cofactor salts may be placed into solution at working concentrations and stored according to the methods of the present invention.

Detergents

At least one detergent may be included as a component of the present compositions, to provide for both increased stability and activity of the component enzymes. Nonionic detergents are preferred, to maintain a balanced ionic strength and prevent chelation of cofactors and aggregation
20 or inactivation of proteins. Particularly preferred as detergents are TRITON X-100®, Brij 35, Tween 20 and Nonidet P-40 (NP-40), although other nonionic surfactants and mixtures thereof may also be used in the present compositions. These detergents are available commercially from sources such as Sigma Chemical Company (Saint Louis, Missouri), usually as concentrated aqueous solutions or in powder form. Once obtained, the detergents may be placed into solution at working
25 concentrations and stored according to the methods of the present invention.

Formulating the Reagent Compositions

Once the reagent components have been obtained, they are mixed at working concentrations to form a solution suitable for immediate use with or without dilution or addition of further reagents.
30 The water used in the formulations of the present invention is preferably distilled, deionized and sterile filtered (through a 0.1-0.2 micrometer filter), and is free of contamination by DNase and RNase enzymes. Such water is available commercially, for example from Sigma Chemical Company

(Saint Louis, Missouri), or may be made as needed according to methods well known to those skilled in the art.

Although the components of the present compositions may be admixed in any sequence, it is often preferable to first dissolve the buffer(s) and cofactor salts in water and to adjust the pH of the solution prior to addition of the remaining components. In this way, the pH-sensitive components (particularly the enzymes, ddNTPs and dNTPs) will be less subject to acid- or alkaline-hydrolysis during formulation.

To formulate the buffered salts solution, a buffer salt which is preferably a salt of tris(hydroxymethyl)aminomethane (TRIS®), and most preferably the hydrochloride salt thereof, is combined with a sufficient quantity of water to yield a solution having a TRIS® concentration of 5-150 millimolar, preferably 10-60 millimolar, and most preferably about 20-60 millimolar. To this solution, a salt of magnesium (preferably either the chloride or sulfate salt thereof) may be added to provide a working concentration thereof of 1-10 millimolar, preferably 1.5-5 millimolar, and most preferably about 1.5-2 millimolar. A salt of potassium (most preferably potassium chloride) may also be added to the solution, at a working concentration of 10-100 millimolar and most preferably about 50 millimolar. An ammonium salt, for example ammonium sulfate, may also be added to the mixture, at a working concentration of 2-50 millimolar, preferably 10-30 millimolar and most preferably 18 millimolar. Combinations of ammonium sulfate and potassium chloride (or other salts) may also be used in formulating the compositions of the present invention. A small amount of a salt of ethylenediaminetetraacetate (EDTA) may also be added (preferably about 0.1 millimolar), although inclusion of EDTA does not appear to be essential to the function or stability of the compositions of the present invention. After addition of all buffers and salts, this buffered salt solution is mixed well until all salts are dissolved, and the pH is adjusted using methods known in the art to a pH value of 7.4 to 9.2, preferably 8.0 to 9.0, and most preferably about 8.3 for compositions to be used in amplification or sequencing of nucleotide fragments up to about 5-6 kilobases in size (hereinafter referred to as "standard compositions"), and about 8.9 for compositions to be used for amplification or sequencing of nucleotide fragments larger than about 5-6 kilobases in size (hereinafter referred to as "large sequence compositions").

To the buffered salt solution, the remaining components of the present composition are added. It is well known in the field that the addition of one or more detergents to an aqueous buffer will aid in the subsequent solubilization of added proteins. Accordingly, at least one nonionic detergent such as TRITON X-100® (preferably at a working concentration of 0.1-1%), Brij 35

(preferably at a concentration of 0.01-1% and most preferably of about 0.1%) or Nonidet P-40 (NP-40, preferably as an admixture with a concentration of 0.004-1%, and most preferably in admixture with Tween 20 at a working concentration of 0.1% for standard compositions and 0.02% for large sequence compositions) may be added to the buffer solution. This detergent is preferably
5 added prior to the introduction of the remaining components into the solution, although the detergent may equivalently be added at any step of formulation. Following formulation, the buffered salt solutions may be filtered through a low protein-binding filter unit that is available commercially (for example from Millipore Corporation, Bedford, Massachusetts) and stored until use.

The remaining components are then added to the solution to formulate the compositions of
10 the present invention. At least one thermostable enzyme (e.g., DNA polymerase) is added and the solution is gently mixed (to minimize protein denaturation). For standard DNA amplification (including via PCR) or sequencing of DNA segments up to about 5-6 kilobases in length, any thermostable DNA polymerase (hereinafter the "primary polymerase") may be used in the standard compositions, although *Taq*, *Tne*, *Tma*, VENT™, DEEPVENT™, *Pfu* or *Pwo* polymerases are
15 preferable at a working concentration in the solution of about 0.1-200 units per milliliter, about 0.1-50 units per milliliter, about 0.1-40 units per milliliter, about 0.1-36 units per milliliter, about 0.1-34 units per milliliter, about 0.1-32 units per milliliter, about 0.1-30 units per milliliter, or about 0.1-20 units per milliliter, and most preferably at a working concentration of about 20 units per milliliter. For amplification of DNA segments larger than 5-6 kilobases in length, large sequence compositions
20 should be formulated by adding to the standard compositions a low concentration of one or more additional thermostable DNA polymerases (hereinafter the "secondary polymerase") containing a 3'-5' exonuclease activity. Particularly suited for this application are VENT™, *Pfu*, *Pwo* or *Tne*, and most preferably DEEPVENT™, DNA polymerases. The additional polymerase(s) should be added to the solution in sufficient quantity to give a final working concentration of about 0.0002-200 units per
25 milliliter, about 0.002-100 units per milliliter, about 0.002-20 units per milliliter, about 0.002-2.0 units per milliliter, about 0.002-1.6 units per milliliter, about 0.002-0.8 units per milliliter, about 0.002-0.4 units per milliliter, or about 0.002-0.2 units per milliliter, most preferably at concentrations of about 0.40 units per milliliter.

It has heretofore been thought that the activity ratios of the primary to secondary polymerases
30 should be maintained at 4:1 - 2000:1 for large sequence amplification (see U.S. Patent No. 5,436,149).

It has now been discovered, however, that in the compositions of the present invention that activity

ratios of the primary to secondary polymerases of 1:1, 1:2, 1:4, 1:5, 1:8, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000 and 1:2000 may be suitable for amplification of large nucleotide sequences.

For nucleic acid sequencing, the reagent compositions may be used as formulated above. For nucleic acid sequencing by the dideoxy method (See U.S. Patent Nos. 4,962,020, 5,173,411 and 5,498,523), however, preferably the mutant *Tne* DNA polymerase shown in SEQ ID NO:2 is added to the reagent compositions. *Tne* polymerase is added to the solution to give a working concentration of about 0.1-10,000 units per milliliter, about 0.1-5000 units per milliliter, about 0.1-2500 units per milliliter, about 0.1-2000 units per milliliter, about 0.1-1500 units per milliliter, about 0.1-1000 units per milliliter, about 0.1-500 units per milliliter, about 0.1-300 units per milliliter, about 0.1-200 units per milliliter, about 0.1-100 units per milliliter, or about 0.1-50 units per milliliter, and most preferably of about 300 units per milliliter.

For dideoxy sequencing, a solution of each ddNTP is also prepared. The base of each solution contains dATP, dCTP, dTTP, 7-deaza-GTP and/or other dNTPs, each at a working concentration of about 10-1000 micromolar, about 10-500 micromolar, about 10-250 micromolar, or about 10-100 micromolar, most preferably at a concentration of about 100 micromolar, in a solution of buffer and chelating salts, for example TRIS®-HCl most preferably at a working concentration of about 10 millimolar (pH about 7.5) and disodium-EDTA most preferably at a concentration of about 0.1 millimolar. To this base, one of the ddNTPs is added to make each of four solutions. Preferably, the sodium or lithium salt of ddATP, ddCTP, ddGTP or ddTTP is added to the solution to give a working concentration of the ddNTP of about 0.5-10 micromolar, about 0.5-8 micromolar, about 0.5-5 micromolar, about 0.5-3 micromolar, about 0.5-2.5 micromolar, or about 0.5-2 micromolar, and most preferably about 2 micromolar. For cycle sequencing applications, the pH of the ddNTP solutions will preferably be about 9.0, and the concentrations of ddNTPs may be lower, preferably about 0.05 to 1.0 micromolar or about 0.05 to 0.8 micromolar, and most preferably about 0.08 to 0.8 micromolar. For some applications, it may be desirable to also incorporate or substitute ddITP, ddUTP, and/or α -thio-dATP into the compositions at approximately the same working concentrations. Thus, four solutions are prepared, each containing one of the four ddNTPs, which are combined with the polymerase compositions of the present invention to carry out the four separate reactions used in dideoxy sequencing. Alternatively, for single-solution sequencing as disclosed in U.S. Patent Nos. 4,962,020 and 5,173,411, the four ddNTPs may be combined into a single solution which is added to the polymerase compositions of the present invention to perform the sequencing reaction.

For nucleic acid amplification, including PCR, dNTP salts are added to the reagent compositions. Preferably, the sodium or lithium salts of dATP, dCTP, dGTP and dTTP are added to the solution to give a working concentration of each dNTP of 10-1000 micromolar, preferably 200-300 micromolar, and most preferably about 200 micromolar. For some applications, it may be desirable to also incorporate or substitute dITP or dUTP into the compositions at the same working concentrations.

To reduce component denaturation, storage of the reagent compositions is preferably in conditions of diminished light, *e.g.*, in amber or otherwise opaque containers or in storage areas with controlled low lighting. The ready-to-use reagent compositions of the present invention are unexpectedly stable at ambient temperature (about 20°-25°C) for about 4-10 weeks, are stable for at least one year upon storage at 4°C, and for at least two years upon storage at -20°C. Surprisingly, storage of the compositions at temperatures below freezing (*e.g.*, -20°C to -70°C), as is traditional with stock solutions of bioactive components, is not necessary to maintain the stability of the compositions of the present invention.

In other preferred embodiments, the compositions of the present invention may be assembled into kits for use in nucleic acid amplification or sequencing. Sequencing kits according to the present invention comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles and the like, wherein a first container means contains a stable composition comprising a mixture of reagents, at working concentrations, which are at least one thermostable DNA polymerase, at least one buffer salt, at least one deoxynucleoside triphosphate and at least one dideoxynucleoside triphosphate. The sequencing kits may further comprise additional reagents and compounds necessary for carrying out standard nucleic sequencing protocols, such as pyrophosphatase, agarose or polyacrylamide media for formulating sequencing gels, and other components necessary for detection of sequenced nucleic acids (See U.S. Patent Nos. 4,962,020 and 5,498,523, which are directed to methods of DNA sequencing).

Similarly, amplification kits according to the present invention comprise carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles and the like, wherein a first container means contains a stable composition comprising a mixture of reagents, at working concentrations, which are at least one thermostable DNA polymerase, at least one buffer salt, and at least one deoxynucleoside triphosphate. The amplification kits encompassed by this aspect of the present invention may further

comprise additional reagents and compounds necessary for carrying out standard nucleic acid amplification protocols (See U.S. Patent Nos. 4,683,195 and 4,683,202, which are directed to methods of DNA amplification by PCR).

5 Use of the Reagent Compositions

The compositions and kits embodied in the present invention will have general utility in any application utilizing nucleic acid sequencing or amplification methodologies. Amplification methods in which the present compositions may be used include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Patent No. 5,455,166; EP 0 684 315),
10 and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822). Nucleic acid sequencing techniques which may employ the present compositions include dideoxy sequencing methods such as those disclosed in U.S. Patent Nos. 4,962,022 and 5,498,523, as well as more complex PCR-based nucleic acid fingerprinting techniques such as Random Amplified Polymorphic DNA (RAPD) analysis (Williams, J.G.K., *et al.*, *Nucl. Acids Res.* 18(22):6531-6535, 1990), Arbitrarily Primed PCR (AP-PCR; Welsh, J., and McClelland, M., *Nucl.*
15 *Acids Res.* 18(24):7213-7218, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anollés *et al.*, *Bio/Technology* 9:553-557, 1991), microsatellite PCR or Directed Amplification of Minisatellite-region DNA (DAMD; Heath, D.D., *et al.*, *Nucl. Acids Res.* 21(24): 5782-5785, 1993), and Amplification Fragment Length Polymorphism (AFLP) analysis (EP 0 534 858; Vos, P., *et al.*, *Nucl.*
20 *Acids Res.* 23(21):4407-4414, 1995; Lin, J.J., and Kuo, J., *FOCUS* 17(2):66-70, 1995). In particular, the compositions and kits of the present invention will be useful in the fields of medical therapeutics and diagnostics, forensics, and agricultural and other biological sciences, in any procedure utilizing thermostable DNA polymerases. Furthermore, the methods by which the compositions of the present invention are formulated may be extendable to all thermostable enzymes
25 or mixtures thereof, and may allow the formulation of ready-to-use compositions of a variety of bioactive enzymes or other proteins that demonstrate increased stability upon extended storage at temperatures above freezing.

The compositions and kits of the invention are particularly useful in methods for amplifying and sequencing nucleic acid molecules. Nucleic acid amplification methods according to this aspect
30 of the invention comprise contacting a nucleic acid molecule to be amplified with one or more of the compositions of the invention, thus providing a population of amplified copies of the nucleic acid molecule. Nucleic acid sequencing methods according to this aspect of the invention comprise

contacting the nucleic acid molecule to be sequenced with one or more of the compositions of the invention. According to these methods, amplification and sequencing of the nucleic acid molecule may be accomplished by any of the above-described amplification and sequencing techniques, most preferably by PCR. The present amplification and sequencing methods are particularly useful for
5 amplification and sequencing of large nucleic acid molecules (*e.g.*, by "long PCR"), preferably nucleic acid molecules that are larger than about 4-8 kilobases in size, more preferably larger than about 5-7 kilobases in size, and most preferably nucleic acid molecules that are larger than about 7 kilobases in size.

10 It will be readily apparent to those of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not
15 intended to be limiting of the invention.

Example 1: Formulation of Standard Compositions

20 As an initial step in formulating stable, ready-to-use reagent compositions for nucleic acid amplification and sequencing, components were mixed in varying amounts as shown in Table 1 to provide 24 different formulations. The pH on all formulations was adjusted to about 8.3. After filtration through a low protein-binding filter, all compositions were formulated with 20 units/ml of *Taq* polymerase and were suitable for use as standard compositions for amplification or sequencing
25 of nucleic acid fragments up to about 5-6 kilobases in size.

TABLE 1. FORMULATIONS OF STANDARD COMPOSITIONS

Formulation Number	1	2	3	4	5	6	7	8
Tris-HCl, pH 8.4	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM
KCl	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
(NH ₄) ₂ SO ₄	0	0	0	0	0	0	0	0
dNTP	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M Na	200 μ M Na	200 μ M Li	200 μ M Li
MgCl ₂	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM
Detergents	0.004% Tween20/NP40	0.1% Tween20/NP40	0.1% Tween20/NP40	0.1% Tween20/NP40	0.1% Tween20/NP40	0.004% Tween20/NP40	0.1% Tween20/NP40	0.004% Tween20/NP40
Taq, U/ml	20	20	20	20	20	20	20	20

9	10	11	12	13	14	15	16	17
20 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM
50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
0	0	0	0	0	0	0	0	0
200 μ M	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M
1.5 mM	3 mM	3 mM	3 mM	3 mM	3 mM	3 mM	3 mM	3 mM
0.1% Tween20/NP40	0.1% Tween20/NP40	0.1% Brij35	0.1% TritonX-100	0.01% Tween20/NP40	0.01% Brij35	0.01% TritonX-100	1% Tween20/NP40	1% Brij35
20	20	20	20	20	20	20	20	20

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18	19	20	21	22	23	24
20 mM	20 mM	20 mM	20 mM	20 mM	50 mM (pH 9.0)	20 mM (pH 8.8)
50 mM	50 mM	50 mM	50 mM	50 mM	0	10 mM
0	0	0	0	0	20 mM	10 mM
200 μ M	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M	200 μ M
3 mM	3 mM	3 mM	3 mM	3 mM	1.5 mM	2 mM
1% TritonX-100	none	0.1% Tween20/NP40	0.1% Tween20/NP40	0.1% Tween20/NP40	0.1% Tween20/NP40	0.1% TritonX100
20	20	20	20	20	20	20

Example 2: Stability of Standard Compositions

To examine the stability of the standard compositions formulated in Example 1, samples of each formulation were aliquoted and stored, under diminished light, at ambient temperature (about 5 20°-25°C), 4°C, -20°C and -70°C. Samples of each formulation from each temperature were taken daily for the first week, and weekly thereafter, and used in stability assays. These stability assays were performed by amplifying, via standard PCR, suboptimal amounts of human genomic DNA as a template using a template titration (if few samples were to be compared in a time point) or a fixed amount of template (if a larger number of samples were to be compared). To the desired amount of 10 template DNA in a given formulation, 10 picomoles of primer was added, and the reaction mixtures were subjected to 35 cycles of PCR of 30 seconds at 94°C, 30 seconds at 55°C and 1 minute/kilobase at 72°C. A portion of each reaction was then subjected to agarose gel electrophoresis and visualized by ethidium bromide fluorescence under ultraviolet illumination.

The results of these assays indicated that certain of the formulations demonstrated enhanced 15 stability upon storage. As shown in Figure 1, after about three months of storage at 4°C, formulations 15 and 19 had completely lost enzymatic activity, as evidenced by an absence of bands in the lanes corresponding to these samples. At this same time point, however, formulations 10-14, 16-18 and 20-24 demonstrated about the same levels of enzymatic activity as a freshly made ("control") formulation.

20 Storage temperature was also found to have a significant effect upon the stability of the formulations, even within a given formulation. As shown in Figure 2, when samples of formulation 4 were examined after about 15 weeks of storage at either 4°C or about 20-25°C, the samples stored at 4°C retained full enzymatic activity when compared to a control sample. Those stored at about 20-25°C, however, had lost some activity, as indicated by the lower yields of the target fragment 25 obtained at all template concentrations.

The results for all of the formulations at the various storage temperatures are summarized in Table 2.

TABLE 2. STABILITY OF STANDARD COMPOSITIONS

Formulation Number ¹	1	2	3	4	5	6	7	8
Storage Temperature, °C								
20-25	< 12 weeks	< 12 weeks	< 15 weeks	< 12 weeks	nd ²	nd	nd	nd
4	> 26 weeks	> 26 weeks	> 56 weeks	> 27 weeks	> 8 weeks	> 8 weeks	> 8 weeks	> 8 weeks
-20	> 13 weeks	> 13 weeks	nd	> 15 weeks	nd	nd	nd	nd
-70	nd	nd	nd	> 15 weeks	nd	nd	nd	nd
9	10	11	12	13	14	15	16	17
nd	< 10 weeks	< 6 weeks	< 6 weeks	< 12 weeks	< 10 weeks	< 7 days	< 12 weeks	< 6 weeks
> 40 weeks	> 26 weeks	> 26 weeks	> 26 weeks	> 26 weeks	> 26 weeks	< 1 day	> 26 weeks	> 26 weeks
> 10 weeks	> 26 weeks	> 26 weeks	> 26 weeks	> 26 weeks	> 26 weeks	< 7 days	> 26 weeks	> 26 weeks
nd	nd	nd	nd	nd	nd	nd	nd	nd
18	19	20	21	22	23	24		
< 6 weeks	0 days	< 10 weeks	< 10 weeks	< 12 weeks	< 6 weeks	< 12 weeks		
> 26 weeks	0 days	> 26 weeks	> 26 weeks	> 13 weeks	> 26 weeks	> 26 weeks		
> 26 weeks	0 days	> 26 weeks	> 26 weeks	> 26 weeks	> 26 weeks	> 26 weeks		
nd	nd	nd	nd	nd	nd	nd		

¹ Formulation numbers correspond to those in Table 1; ² nd = not done.

These results indicate that several of the compositions unexpectedly maintained enzymatic activity for 6-12 weeks upon storage at 20-25°C, and for over one year at 4°C. Formulation 19, however, had completely lost activity within 24 hours of formulation. Formulation 15 also exhibited a rapid loss of activity.

5 For further analysis of the stability of these ready-to-use compositions, several formulations were stored at 20-25°C or at 4°C for up to six months, with samples taken monthly for stability assays performed by a determination of polymerase unit activity. The results of these assays are summarized in Tables 3 and 4.

10 **TABLE 3. STABILITY OF STANDARD COMPOSITIONS
(PERCENTAGE OF ENZYME ACTIVITY REMAINING)
UPON STORAGE AT 20-25°C**

Formulation No. ¹	1 Month	2 Months	3 Months	4 Months
10	89	65	nd ²	nd
11	106	3	nd	nd
12	106	91	nd	nd
13	93	72	nd	nd
14	91	76	nd	nd
15	78	63	nd	nd
16	94	85	nd	nd
17	89	90	nd	nd
18	90	84	nd	nd
19	0	0	nd	nd
20	88	81	72	77
21	nd	103	nd	nd
22	97	84	nd	nd
23	83	77	nd	nd
24	81	106	nd	nd

¹ Formulation numbers correspond to those in Table 1.

² nd = not done

**TABLE 4. STABILITY OF STANDARD COMPOSITIONS
(PERCENTAGE OF ENZYME ACTIVITY REMAINING)
UPON STORAGE AT 4°C**

Formulation No. ¹	1 Month	2 Months	4 Months	5 Months	6 Months
10	84	86	98	nd ²	105
11	94	97	98	nd	nd
12	94	97	106	nd	nd
13	86	93	93	nd	nd
14	85	91	105	nd	nd
15	89	89	98	nd	nd
16	88	103	104	nd	nd
17	83	94	91	nd	nd
18	90	97	99	nd	nd
19	95	59	nd	nd	38
20	97	100	94	94	103
21	93	97	100	nd	nd
22	100	109	35	nd	nd
23	94	97	89	nd	107
24	93	94	97	nd	nd

¹ Formulation numbers correspond to those in Table 1.

² nd - not done

Several of the formulations were stable upon storage at 20-25°C, most notable formulation 20 which retained >70% of its enzymatic activity even after storage for four months at this temperature. As described earlier, formulation 19 had completely lost activity within the first month, as determined by the PCR assay. Interestingly, however, as determined by the polymerase unit activity assay, formulation 19 was stable for one month when stored at 4°C but had lost substantial activity by the second month of storage at this temperature (Table 4). Formulation 20, shown previously to be stable upon extended storage at 20-25°C, was stable upon storage at 4°C for at least six months.

Taken together, these results indicate that the compositions of the present invention are readily suitable for use in nucleic acid amplification reactions, and demonstrate extended stability upon storage at 20-25°C or 4°C.

5 **Example 3: Formulation and Stability of Large Sequence Compositions**

For use in amplification and sequencing of nucleic acid fragments larger than 5-6 kilobases, it has been suggested as described above that a mixture of *Taq* and VENT™ or DEEPVENT™ polymerases (U.S. Patent No. 5,436,149; Barnes, *Id.*), or of *Tth* and either *Tli*, *Pyrococcus* or *Tma* (U.S. Patent No. 5,512,462), be used. Accordingly, *Taq* and DEEPVENT™ DNA polymerases
10 were formulated, at activity ratios of 100:1 (for samples 1-3) or 50:1 (for sample 4) into solutions containing the buffer salts, cofactors and detergents shown in Table 5. Each of these formulations was adjusted to about pH 9.1, which is optimal for the activity of DEEPVENT™ DNA polymerase (Bej and Mahbubani, *Id.*). Samples were then stored at about 20-25°C or at 4°C and assayed weekly
15 for 12 weeks, and monthly thereafter, in stability assays in which a 20 kilobase target in 100 nanograms of human genomic template was amplified by PCR. Reaction mixtures included 10 picomoles of primer and were subjected to 35 PCR cycles of 30 seconds at 94°C, 30 seconds at 62°C and 1 minute/kilobase at 68°C. Portions of the reaction were subjected to agarose gel electrophoresis and were visualized by ethidium bromide fluorescence under ultraviolet illumination,
20 as shown above for Figures 1 and 2. Results are summarized in Table 5.

TABLE 5. STABILITY OF LARGE SEQUENCE COMPOSITIONS

Formulation No.	Formulation	Stability at:	
		20-25°C	4°C
1	66 mM Tris-SO ₄ (pH 9.1)	< 12 weeks	11 months
	19.8 mM (NH ₄) ₂ SO ₄		
	2.2 mM MgSO ₄		
	22 units/ml <i>Taq</i> DNA Polymerase		
	0.22 units/ml DEEPVENT DNA Polymerase		
	0.11% Tween-20		
	0.011% NP-40		
2	66 mM Tris-SO ₄ (pH 9.1)	< 12 weeks	> 11 months
	19.8 mM (NH ₄) ₂ SO ₄		
	2.2 mM MgSO ₄		
	24.42 units/ml <i>Taq</i> DNA Polymerase		
	0.242 units/ml DEEPVENT DNA Polymerase		
	0.066% Tween-20		
	0.066% NP-40		
3	66 mM Tris-SO ₄ (pH 9.1)	nd ¹	11 months
	19.8 mM (NH ₄) ₂ SO ₄		
	2.2 mM MgSO ₄		
	22 units/ml <i>Taq</i> DNA Polymerase		
	0.22 units/ml DEEPVENT DNA Polymerase		
	0.01% Tween-20		
	0.01% NP-40		
4	66 mM Tris-SO ₄ (pH 9.1)	nd ¹	11 months
	19.8 mM (NH ₄) ₂ SO ₄		
	2.2 mM MgSO ₄		
	22 units/ml <i>Taq</i> DNA Polymerase		
	0.44 units/ml DEEPVENT DNA Polymerase		
	0.01% Tween-20		
	0.01% NP-40		

¹ nd = not done

Upon storage at ambient temperature (20°-25°C), all of the formulations were stable for 6-12 weeks. Storage of these formulations at 4°C provided enhanced stability of over 11 months. Similar results may be obtained with formulations in which *Taq* and *Tne* DNA polymerases were used in an activity ratio of 1:1, 1:2, 1:4, 1:5, 1:8, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000 or 1:2000. These results indicate that the large sequence compositions of the present invention are readily suitable for use in amplification of nucleic acid sequences larger than 5-6 kilobases and demonstrate extended stability upon storage at 20° to 25°C, or at 4°C.

Example 4: Combinations of *Thermus flavus* (*Tfl*) DNA Polymerase and *Thermotoga neapolitana* (*Tne*) DNA Polymerase

To examine other DNA polymerase compositions for their utility in amplification of nucleic acid molecules, a mixture of *Tfl* and *Tne* DNA polymerases, at a 1:1 ratio, was used to amplify the 2.7 kilobase Puc19 plasmid. Amplification reactions were in a 50 µl final volume in buffer containing 1 mM magnesium acetate. 80 pg of Puc19 linearized by treatment with *AdII* was used as the template, and was contacted with 1 µl of enzyme mixture. PCR conditions were 1 min at 94°C, followed by 35 cycles of 94°C for 30 seconds/60°C for 30 seconds/68°C for 5 minutes.

Upon analysis of the amplification products by gel electrophoresis, this composition comprising *Tfl* and *Tne* DNA polymerases was found to efficiently amplify the 2.7 kilobase Puc19 plasmid. The efficiency of amplification was comparable to amplification of Puc19 using 1 µl of *Taq* DNA polymerase.

Example 5: Amplification of Genomic DNA Using *Tfl*/*Tne* Compositions

Having demonstrated that compositions comprising *Tfl* and *Tne* DNA polymerases efficiently amplify plasmid-sized nucleic acid molecules, these compositions were examined for their ability to amplify nucleic acid molecules from genomic DNA templates. Six different primer sets were constructed (ranging in size from 0.25 to 4.1 kilobases) and used to amplify the human β-globin gene from a genomic DNA template from the K562 human leukemia cell line. Each reaction was performed in a volume of 50 µl comprising template at 40 ng/reaction, and *Tfl*/*Tne* mixture at either 0.5 unit/reaction or 1 unit/reaction (*Tfl* and *Tne* at a 1:1 ratio in both mixtures). PCR conditions

were 1 min at 94°C, followed by 35 cycles of 94°C for 30 seconds/55°C for 30 second/68°C for 5 minutes.

Upon analysis of the amplification products by gel electrophoresis, efficient amplification was observed for all primers. The sizes of the amplification products produced using the different primers were 0.25 kilobase, 0.7 kilobase, 1.1 kilobases, 2.0 kilobases and 4.1 kilobases. These results demonstrate that the *Tfl/Tne* compositions efficiently amplify nucleic acid molecules derived from genomic DNA templates.

Example 6: Use of Various Enzyme Ratios in DNA Polymerase Compositions

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To determine the efficacy of different enzyme ratios in nucleic acid amplification, a composition comprising a higher amount of *Tne* (3' exo+) DNA polymerase than *Tfl* (3' exo-) DNA polymerase was made and tested for its ability to amplify Puc19 and the β -globin gene. A 1:3 mixture of *Tfl/Tne* was made and used to amplify Puc19 under the conditions described in Example 4, and β -globin under the conditions described in Example 5.

15

Upon analysis of the amplification products by gel electrophoresis, efficient amplification was observed for both Puc19 and for β -globin (all template sizes). These results demonstrate that compositions in which a 3' exo+ DNA polymerase (*Tne*) is present in higher quantity than a 3' exo- DNA polymerase (*Tfl*) efficiently amplify nucleic acid molecules derived from plasmid and genomic DNA templates.

20

Example 7: Amplification of Large Nucleic Acid Molecules Using DNA Polymerase Mixtures

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Having demonstrated that compositions comprising mixtures of 3' exo+ and 3' exo- DNA polymerases efficiently amplify plasmid-sized and small genomic nucleic acid molecules, such compositions were examined for their ability to amplify larger nucleic acid molecules. For these studies, mixtures of *Taq* (3' exo+) DNA polymerase and *Tma* (3' exo-) DNA polymerase (ULTma™; LTI, Gaithersburg, Maryland) were prepared. Two sets of mixtures were prepared: one set contained 1 unit of *Taq* and varying amounts of ULTma (0.3 unit, 0.6 unit, 0.8 unit or 1 unit), and the other set contained no *Taq* and only varying amounts of ULTma (0.3 unit, 0.6 unit, 0.8 unit or 1 unit). These compositions were used to amplify 80 mg of human genomic DNA using primers

30

specific for a 7.5 kilobase region of the human β -globin gene. PCR conditions were 1 min at 94°C, followed by 35 cycles of 94°C for 30 seconds/60°C for 30 seconds/68°C for 5 minutes.

Upon analysis of the amplification products by gel electrophoresis, the 7.5 kilobase β -globin fragment was found to be efficiently amplified by all compositions comprising both *Taq* and *Tma* DNA polymerase. Compositions comprising only *Tma* DNA polymerase, however, were unable to amplify this large fragment. In control experiments, compositions comprising only *Taq* DNA polymerase were also unable to amplify this large fragment. These results demonstrate that compositions comprising a mixture of 3' exo+ and 3' exo- DNA polymerases are useful in efficiently amplifying large nucleic acid molecules, particularly in amplifying nucleic acid molecules larger than about 7 kilobases in size.

Example 8: Amplification of Large Nucleic Acid Molecules Using Various Ratios of *Taq* and *Tma* DNA Polymerases

Having demonstrated that compositions comprising mixtures of 3' exo+ (*Taq*) and 3' exo- (*Tma*) DNA polymerases efficiently amplify large nucleic acid molecules, compositions comprising these enzymes in various ratios were made and tested for their abilities to amplify the 7.5 kilobase β -globin gene fragment from Example 7. Two sets of mixtures were prepared: one set contained 1 unit of ULTma DNA polymerase and different amounts (0.25, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 unit) of *Taq* DNA polymerase, and the other set contained 1 unit of *Taq* DNA polymerase and different amounts (1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 3.0, 4.0 or 6.0 units) of ULTma DNA polymerase. Amplification templates, primers and cycling conditions were as described for Example 7.

Upon analysis of the amplification products by gel electrophoresis, efficient amplification of the 7.5 kilobase β -globin gene was observed for the first set of mixtures only in those compositions containing 0.5 to 1.0 unit of *Taq* DNA polymerase and 1.0 unit of ULTma. Compositions in this set containing less than 0.5 unit of *Taq* DNA polymerase did not amplify this fragment. However, all enzyme mixtures in the other set, i.e., compositions comprising 1.0 unit of *Taq* DNA polymerase and 1.0 to 6.0 units of ULTma DNA polymerase, demonstrated efficient amplification of the 7.5 kilobase fragment. In separate experiments, a 13.5 kilobase fragment of the β -globin gene was efficiently amplified using mixtures containing a 1:1 or a 1:2 ratio of *Taq* to ULTma. Together, these results indicate that compositions in which *Tma* DNA polymerase is present in equal or higher quantity than

Taq DNA polymerase efficiently amplify large nucleic acid molecules, particularly those that are larger than about 7-13 kilobases in size.

Example 9: Use of *Taq* and *Tne* DNA Polymerase Mixtures for Long PCR

5 To determine if other DNA polymerases could be used in compositions also comprising *Taq* DNA polymerase in amplification of large nucleic acid molecules, various mixtures of *Taq* and *Tne* DNA polymerases were made. For these experiments, a *Tne* DNA polymerase deletion mutant (5' exo-; 3' exo+) was mixed in amounts ranging from 0.05 to 2.0 units with 1 unit of *Taq* DNA
10 polymerase and used to amplify the 7.5 kilobase β -globin fragment under conditions described for Example 7.

Upon analysis of the amplification products by gel electrophoresis, all of the combinations of *Tne* and *Taq* DNA polymerases were found to efficiently amplify the 7.5 kilobase DNA fragment. These results indicate that compositions comprising combinations of *Taq* and *Tne* DNA polymerases
15 are useful in amplifying large nucleic acid molecules, particularly those larger than about 7 kilobases in size.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art
20 that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative
25 of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
(A) NAME: Life Technologies, Inc.
(B) STREET: 9800 Medical Center Drive
10 (C) CITY: Rockville
(D) STATE: Maryland
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 20850
- 15 (ii) TITLE OF INVENTION: Stable Compositions for Nucleic Acid
Amplification and Sequencing
- (iii) NUMBER OF SEQUENCES: 2
- 20 (iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)
- 25 (vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/689,815
(B) FILING DATE: 14-AUG-1996
- 30 (vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/801,720
(B) FILING DATE: 14-FEB-1997

(2) INFORMATION FOR SEQ ID NO: 1:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 893 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 45
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
- 50 Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala
1 5 10 15
- Tyr Tyr Ala Leu Asp Arg Ser Leu Ser Thr Ser Thr Gly Ile Pro Thr
20 25 30
- 55 Asn Ala Val Tyr Gly Val Ala Arg Met Leu Val Lys Phe Ile Lys Glu
35 40 45

His Ile Ile Pro Glu Lys Asp Tyr Ala Ala Val Ala Phe Asp Lys Lys
 50 55 60
 5 Ala Ala Thr Phe Arg His Lys Leu Leu Val Ser Asp Lys Ala Gln Arg
 65 70 75 80
 Pro Lys Thr Pro Ala Leu Leu Val Gln Gln Leu Pro Tyr Ile Lys Arg
 85 90 95
 10 Leu Ile Glu Ala Leu Gly Phe Lys Val Leu Glu Leu Glu Gly Tyr Glu
 100 105 110
 Ala Asp Asp Ile Ile Ala Thr Leu Ala Val Arg Ala Ala Arg Phe Leu
 115 120 125
 15 Met Arg Phe Ser Leu Ile Thr Gly Asp Lys Asp Met Leu Gln Leu Val
 130 135 140
 20 Asn Glu Lys Ile Lys Val Trp Arg Ile Val Lys Gly Ile Ser Asp Leu
 145 150 155 160
 Glu Leu Tyr Asp Ser Lys Lys Val Lys Glu Arg Tyr Gly Val Glu Pro
 165 170 175
 25 His Gln Ile Pro Asp Leu Leu Ala Leu Thr Gly Asp Asp Ile Asp Asn
 180 185 190
 Ile Pro Gly Val Thr Gly Ile Gly Glu Lys Thr Ala Val Gln Leu Leu
 195 200 205
 30 Gly Lys Tyr Arg Asn Leu Glu Tyr Ile Leu Glu His Ala Arg Glu Leu
 210 215 220
 35 Pro Gln Arg Val Arg Lys Ala Leu Leu Arg Asp Arg Glu Val Ala Ile
 225 230 235 240
 Leu Ser Lys Lys Leu Ala Thr Leu Val Thr Asn Ala Pro Val Glu Val
 245 250 255
 40 Asp Trp Glu Glu Met Lys Tyr Arg Gly Tyr Asp Lys Arg Lys Leu Leu
 260 265 270
 Pro Ile Leu Lys Glu Leu Glu Phe Ala Ser Ile Met Lys Glu Leu Gln
 275 280 285
 45 Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu Ile Val Lys Asp His
 290 295 300
 50 Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys Glu Val Pro Ser Phe
 305 310 315 320
 Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro Phe Asn Cys Glu Ile
 325 330 335
 55 Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr Ala Tyr Tyr Ile Pro
 340 345 350

	Leu His His Arg Asn Ala His Asn Leu Asp Glu Thr Leu Val Leu Ser	355	360	365
5	Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser Lys Ile Val Gly Gln	370	375	380
	Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val Lys Gly Ile Ser Pro	385	390	395
10	Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro	405	410	415
	Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser Leu Lys Phe Leu Gly	420	425	430
15	Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser Phe Ser Ser Pro Leu	435	440	445
	Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp Lys Ala Ala Glu Tyr	450	455	460
20	Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu Tyr Lys Ile Leu Ser	465	470	475
	Met Lys Leu His Glu Ala Glu Leu Glu Asn Val Phe Tyr Arg Ile Glu	485	490	495
	Met Pro Leu Val Asn Val Leu Ala Arg Met Glu Phe Asn Trp Val Tyr	500	505	510
30	Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys	515	520	525
	Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro Phe	530	535	540
	Asn Ile Asn Ser Pro Lys Gln Val Ser Asn Ile Leu Phe Glu Lys Leu	545	550	555
40	Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Asp Tyr Ser Thr	565	570	575
	Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu His Glu Ile Val Pro	580	585	590
45	Leu Ile Leu Glu Phe Arg Lys Ile Leu Lys Leu Lys Ser Thr Tyr Ile	595	600	605
	Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr Gly Arg Phe His Ala	610	615	620
	Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp	625	630	635
55	Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile	645	650	655

Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp Trp Ile Val Ser Ala
 660 665 670
 5 Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp
 675 680 685
 Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu
 690 695 700
 10 Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu Glu Val Asn Glu Glu
 705 710 715 720
 Met Arg Arg Val Gly Lys Met Val Asn Phe Ser Ile Ile Tyr Gly Val
 725 730 735
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 755 760 765
 Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu Lys Gly Tyr Val Arg
 770 775 780
 25 Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg Asp
 785 790 795 800
 Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro Ile
 805 810 815
 30 Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala Met Ile Asp Ile Asp
 820 825 830
 Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg Met Ile Ile Gln Val
 835 840 845
 His Asp Glu Leu Val Phe Glu Val Pro Asp Glu Glu Lys Glu Glu Leu
 850 855 860
 40 Val Asp Leu Val Lys Asn Lys Met Thr Asn Val Val Lys Leu Ser Val
 865 870 875 880
 Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser Trp Ser
 885 890
 45

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 893 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5 Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala
 1 5 10 15
 Tyr Tyr Ala Leu Asp Arg Ser Leu Ser Thr Ser Thr Gly Ile Pro Thr
 20 25 30
 10 Asn Ala Val Tyr Gly Val Ala Arg Met Leu Val Lys Phe Ile Lys Glu
 35 40 45
 His Ile Ile Pro Glu Lys Asp Tyr Ala Ala Val Ala Phe Asp Lys Lys
 50 55 60
 15 Ala Ala Thr Phe Arg His Lys Leu Leu Val Ser Asp Lys Ala Gln Arg
 65 70 75 80
 Pro Lys Thr Pro Ala Leu Leu Val Gln Gln Leu Pro Tyr Ile Lys Arg
 85 90 95
 20 Leu Ile Glu Ala Leu Gly Phe Lys Val Leu Glu Leu Glu Gly Tyr Glu
 100 105 110
 25 Ala Asp Asp Ile Ile Ala Thr Leu Ala Val Arg Ala Ala Arg Phe Leu
 115 120 125
 Met Arg Phe Ser Leu Ile Thr Gly Asp Lys Asp Met Leu Gln Leu Val
 130 135 140
 30 Asn Glu Lys Ile Lys Val Trp Arg Ile Val Lys Gly Ile Ser Asp Leu
 145 150 155 160
 Glu Leu Tyr Asp Ser Lys Lys Val Lys Glu Arg Tyr Gly Val Glu Pro
 165 170 175
 35 His Gln Ile Pro Asp Leu Leu Ala Leu Thr Gly Asp Asp Ile Asp Asn
 180 185 190
 40 Ile Pro Gly Val Thr Gly Ile Gly Glu Lys Thr Ala Val Gln Leu Leu
 195 200 205
 Gly Lys Tyr Arg Asn Leu Glu Tyr Ile Leu Glu His Ala Arg Glu Leu
 210 215 220
 45 Pro Gln Arg Val Arg Lys Ala Leu Leu Arg Asp Arg Glu Val Ala Ile
 225 230 235 240
 Leu Ser Lys Lys Leu Ala Thr Leu Val Thr Asn Ala Pro Val Glu Val
 245 250 255
 50 Asp Trp Glu Glu Met Lys Tyr Arg Gly Tyr Asp Lys Arg Lys Leu Leu
 260 265 270
 55 Pro Ile Leu Lys Glu Leu Glu Phe Ala Ser Ile Met Lys Glu Leu Gln
 275 280 285

	Leu Tyr Glu Glu Ala Glu Pro Thr Gly Tyr Glu Ile Val Lys Asp His	
	290	295 300
5	Lys Thr Phe Glu Asp Leu Ile Glu Lys Leu Lys Glu Val Pro Ser Phe	
	305	310 315 320
	Ala Leu Asp Leu Glu Thr Ser Ser Leu Asp Pro Phe Asn Cys Glu Ile	
		325 330 335
10	Val Gly Ile Ser Val Ser Phe Lys Pro Lys Thr Ala Tyr Tyr Ile Pro	
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	Leu His His Arg Asn Ala His Asn Leu Asp Glu Thr Leu Val Leu Ser	
		355 360 365
15	Lys Leu Lys Glu Ile Leu Glu Asp Pro Ser Ser Lys Ile Val Gly Gln	
		370 375 380
20	Asn Leu Lys Tyr Asp Tyr Lys Val Leu Met Val Lys Gly Ile Ser Pro	
		385 390 395 400
	Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro	
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25	Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser Leu Lys Phe Leu Gly	
		420 425 430
	Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser Phe Ser Ser Pro Leu	
		435 440 445
30	Phe Gly Phe Ser Phe Ala Asp Val Pro Val Asp Lys Ala Ala Glu Tyr	
		450 455 460
35	Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu Tyr Lys Ile Leu Ser	
		465 470 475 480
	Met Lys Leu His Glu Ala Glu Leu Glu Asn Val Phe Tyr Arg Ile Glu	
		485 490 495
40	Met Pro Leu Val Asn Val Leu Ala Arg Met Glu Phe Asn Trp Val Tyr	
		500 505 510
	Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys	
		515 520 525
45	Leu Glu Glu Leu Ala Glu Lys Ile Tyr Gln Ile Ala Gly Glu Pro Phe	
		530 535 540
50	Asn Ile Asn Ser Pro Lys Gln Val Ser Asn Ile Leu Phe Glu Lys Leu	
		545 550 555 560
	Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Asp Tyr Ser Thr	
		565 570 575
55	Arg Ile Glu Val Leu Glu Glu Ile Ala Asn Glu His Glu Ile Val Pro	
		580 585 590

Leu Ile Leu Glu Phe Arg Lys Ile Leu Lys Leu Lys Ser Thr Tyr Ile
 595 600 605
 5 Asp Thr Leu Pro Lys Leu Val Asn Pro Lys Thr Gly Arg Phe His Ala
 610 615 620
 Ser Phe His Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp
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 10 Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile
 645 650 655
 Arg Lys Ala Ile Val Pro Gln Asp Pro Asp Trp Trp Ile Val Ser Ala
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 15 Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp
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 Glu Asn Leu Val Lys Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu
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 20 Thr Ala Ser Arg Ile Tyr Asn Val Lys Pro Glu Glu Val Asn Glu Glu
 705 710 715 720
 25 Met Arg Arg Val Gly Lys Met Val Asn Tyr Ser Ile Ile Tyr Gly Val
 725 730 735
 Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Ile Pro Val Lys Glu Ala
 740 745 750
 30 Glu Lys Met Ile Ile Ser Tyr Phe Thr Leu Tyr Pro Lys Val Arg Ser
 755 760 765
 Tyr Ile Gln Gln Val Val Ala Glu Ala Lys Glu Lys Gly Tyr Val Arg
 770 775 780
 35 Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg Asp
 785 790 795 800
 40 Lys Asn Thr Gln Ser Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro Ile
 805 810 815
 Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala Met Ile Asp Ile Asp
 820 825 830
 45 Glu Glu Leu Arg Lys Arg Asn Met Lys Ser Arg Met Ile Ile Gln Val
 835 840 845
 His Asp Glu Leu Val Phe Glu Val Pro Asp Glu Glu Lys Glu Glu Leu
 850 855 860
 50 Val Asp Leu Val Lys Asn Lys Met Thr Asn Val Val Lys Leu Ser Val
 865 870 875 880
 55 Pro Leu Glu Val Asp Ile Ser Ile Gly Lys Ser Trp Ser
 885 890

What Is Claimed Is:

1. A stable composition comprising a mixture of reagents at working concentrations, wherein said reagents are at least one thermostable enzyme and at least one buffer salt.

2. A stable composition for nucleic acid amplification comprising a mixture of reagents, wherein said reagents are at least one thermostable DNA polymerase, at least one buffer salt and at least one deoxynucleoside triphosphate.

3. A stable composition for nucleic acid sequencing comprising a mixture of reagents, wherein said reagents are at least one thermostable DNA polymerase, at least one deoxynucleoside triphosphate, at least one dideoxynucleoside triphosphate and at least one buffer salt.

4. The stable composition of claim 2 or claim 3, wherein said reagents are present at working concentrations.

5. The composition of claim 2 or claim 3, wherein said thermostable DNA polymerase is selected from the group of thermostable DNA polymerases consisting of a *Taq* DNA polymerase, a *Tne* DNA polymerase, a *Tma* DNA polymerase, and mutants thereof.

6. The composition of claim 2 or claim 3, wherein said thermostable DNA polymerase is selected from the group of thermostable DNA polymerases consisting of a *Pfu* DNA polymerase, a *Pwo* DNA polymerase, VENT™ DNA polymerase, DEEPVENT™ DNA polymerase, and mutants thereof.

7. The composition of claim 5, wherein said mixture further comprises DEEPVENT™ DNA polymerase or VENT™ DNA polymerase.

8. The composition of claim 5, wherein the concentration of *Taq* DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

9. The composition of claim 8, wherein the concentration is about 20 units per milliliter.

10. The composition of claim 5, wherein the concentration of *Tne* DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

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11. The composition of claim 10, wherein the concentration is about 20 units per milliliter.

12. The composition of claim 5, wherein the concentration of *Tma* DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

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13. The composition of claim 12, wherein the concentration is about 20 units per milliliter.

14. The composition of claim 6, wherein the concentration of VENT™ DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

15

15. The composition of claim 14, wherein the concentration is about 20 units per milliliter.

16. The composition of claim 6, wherein the concentration of DEEPVENT™ DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

20

17. The composition of claim 16 wherein the concentration is about 20 units per milliliter.

18. The composition of claim 6, wherein the concentration of *Pfu* DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

25

19. The composition of claim 18 wherein the concentration is about 20 units per milliliter.

20. The composition of claim 6, wherein the concentration of *Pwo* DNA polymerase or mutant thereof is about 0.1 to 200 units per milliliter.

30

21. The composition of claim 20 wherein the concentration is about 20 units per milliliter.

22. The composition of claim 7, wherein the concentration of DEEPVENT™ DNA polymerase or VENT DNA polymerase is about 0.002 to 200 units per milliliter.

23. The composition of claim 22, wherein the concentration is about 0.40 units per
5 milliliter.

24. The composition of claim 2 or claim 3, wherein said DNA polymerase retains at least 90% of the enzymatic activity for at least four weeks when stored at about 20°C to 25°C.

10 25. The composition of claim 5, wherein said DNA polymerase retains at least 90% of the enzymatic activity for at least one year when stored at about 4°C.

26. The composition of claim 2 or claim 3, further comprising a magnesium salt.

15 27. The composition of claim 2 or claim 3, further comprising at least one nonionic detergent.

28. The composition of claim 2 or claim 3, wherein the concentration of said deoxynucleoside triphosphate is about 200 to about 300 micromolar.

20 29. The composition of claim 3, wherein the concentration of said dideoxynucleoside triphosphate is about 0.08 to about 5 micromolar.

30. A nucleic acid amplification kit comprising one or more containers, wherein a first
25 container contains a stable composition comprising a mixture of reagents, wherein said reagents are at least one thermostable DNA polymerase, at least one buffer salt, and at least one deoxynucleoside triphosphate.

31. A nucleic acid sequencing kit comprising one or more containers, wherein a first
30 container contains a stable composition comprising a mixture of reagents, wherein said reagents are at least one thermostable DNA polymerase, at least one buffer salt, at least one deoxynucleoside triphosphate and at least one dideoxynucleoside triphosphate.

32. The kit of claim 30 or 31, wherein said reagents are present at working concentrations.

33. A method of amplifying a nucleic acid molecule comprising contacting said nucleic acid molecule with the composition of claim 2.

34. A method of amplifying a nucleic acid molecule comprising contacting said nucleic acid molecule with a composition selected from the group consisting of :

a composition comprising a thermostable 3' exo+ DNA polymerase and a thermostable 3' exo- DNA polymerase wherein the concentrations of said 3' exo+ DNA polymerase and of said 3' exo- DNA polymerase are equal, and

a composition comprising a thermostable 3' exo+ DNA polymerase and a thermostable 3' exo- DNA polymerase wherein the concentration of said 3' exo+ DNA polymerase is higher than the concentration of said 3' exo- DNA polymerase.

35. A method of sequencing a nucleic acid molecule comprising contacting said nucleic acid molecule with the composition of claim 3.

36. A method of sequencing a nucleic acid molecule comprising contacting said nucleic acid molecule with a composition selected from the group consisting of :

a composition comprising a thermostable 3' exo+ DNA polymerase and a thermostable 3' exo- DNA polymerase wherein the concentrations of said 3' exo+ DNA polymerase and of said 3' exo- DNA polymerase are equal, and

a composition comprising a thermostable 3' exo+ DNA polymerase and a thermostable 3' exo- DNA polymerase wherein the concentration of said 3' exo+ DNA polymerase is higher than the concentration of said 3' exo- DNA polymerase.

37. The method of any one of claims 33-36, wherein said nucleic acid molecule is larger than about 4 kilobases in size.

38. The method of claim 37, wherein said nucleic acid molecule is larger than about 7 kilobases in size.

39. The method of claim 38, wherein said nucleic acid molecule is larger than about 8 kilobases in size.

40. A nucleic acid molecule amplified by the method of claim 33 or claim 34.

41. The nucleic acid molecule of claim 40, wherein said nucleic acid molecule is larger than about 4 kilobases in size.

42. The nucleic acid molecule of claim 41, wherein said nucleic acid molecule is larger than about 7 kilobases in size.

43. The nucleic acid molecule of claim 41, wherein said nucleic acid molecule is larger than about 8 kilobases in size.

Figure 1

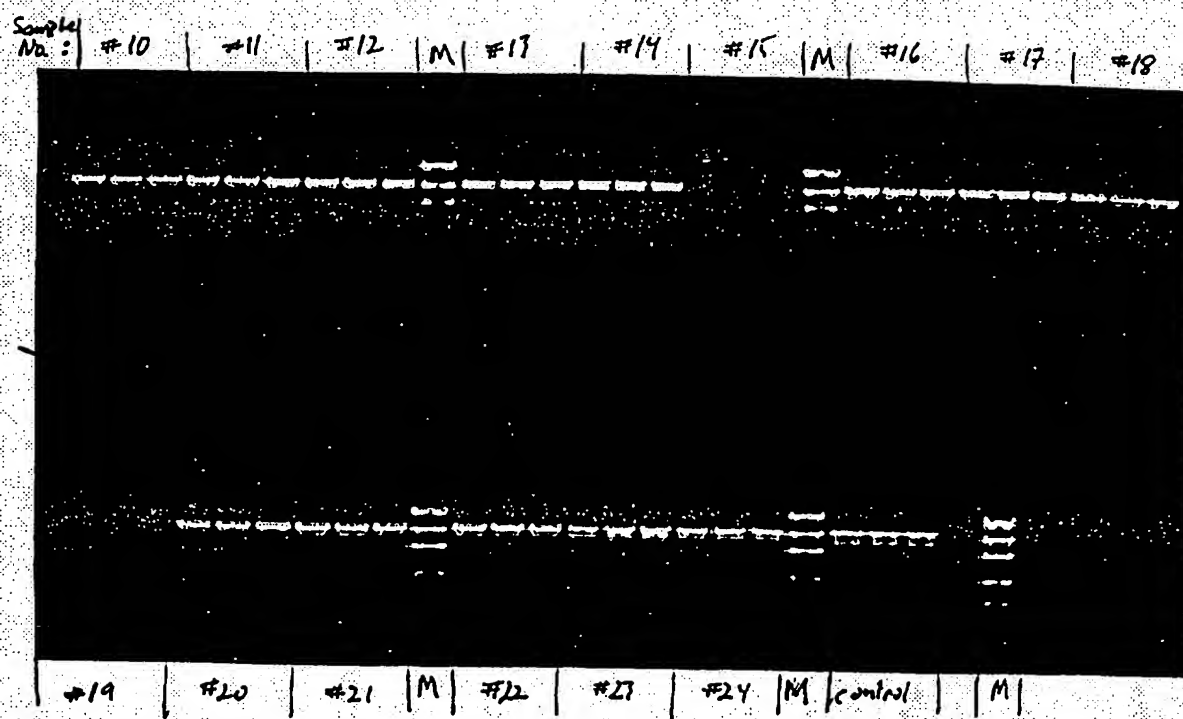
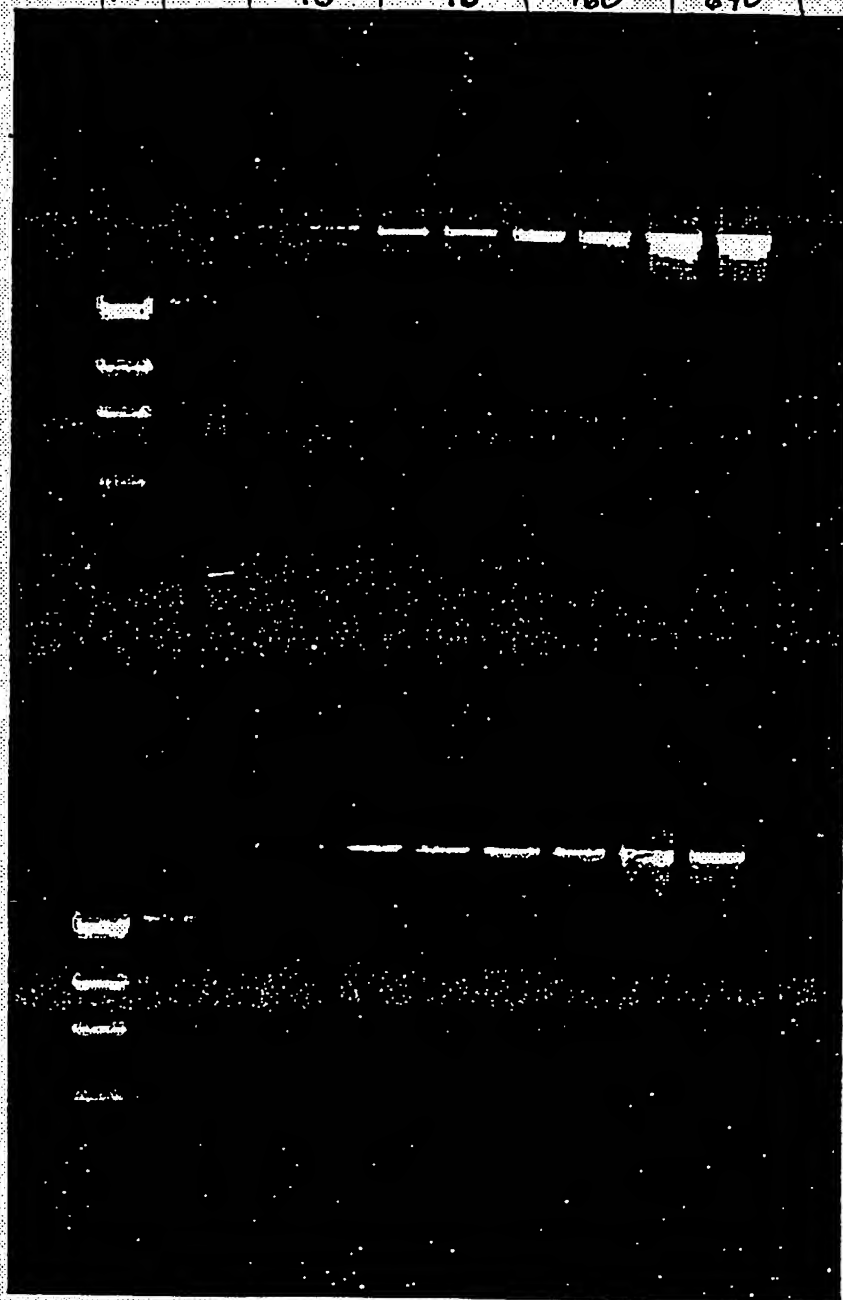


Figure 2

Amount of template/reaction, ng

| M | | 10 | | 40 | | 160 | | 640 |



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14266

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12P 19/34

US CL : 435/91.1, 91.2; 536/22.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.1, 91.2; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, NTIS, AGRICOLA, CAB ABSTRACTS, EMBASE, LIFE SCIENCES, JICST, MEDLINE, PASCAL, DERWENT, CA, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----- Y	HUANG et al. Fidelity and Predominant Mutations Produced by Deep Vent Wild-Type and Exonuclease-Deficient DNA Polymerases during In Vitro DNA Amplification. DNA and Cell Biology. 1996, Vol. 17, No. 7, pages 589-594, especially page 590.	1, 2, 4 ----- 3, 5-34, 37-43
X ----- Y	FLAMAN et al. A Rapid PCR Fidelity Assay. Nucleic Acids Research. 1994, Vol. 22, No. 15, pages 3259-3260, especially page 3259.	1, 2, 4 ----- 3, 5-34, 37-43
X ----- Y	BARNES, W.M. PCR Amplification of up to 35-kb DNA with High Fidelity and High Yield from Lambda Bacteriophage Templates. Proc. Natl. Acad. Sci. March 1994, Vol. 91, pages 2216-2220, especially pages 2216 and 2217.	1, 2, 4 ----- 3, 5-34, 37-43

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O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 SEPTEMBER 1997

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Facsimile No. (703) 305-3230

Authorized officer

PAUL E. TRAN, Ph.D.

Telephone No. (703) 305-0196

INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 5,489,523 A (MATHUR) 06 February 1996, columns 14-19.	1, 2, 4 ----- 3, 5-43
X ----- Y	US 5,466,591 A (ABRAMSON et al.) 14 November 1995, columns 19-23 and 29-33.	1, 2, 4 ----- 3, 5-43
X ----- Y	US 5,420,029 A (GELFAND et al.) 30 May 1995, columns 21-23 and 38.	1, 2, 4 ----- 3, 5-34, 37-43
X ----- Y	WO 9516028 A1 (STRATAGENE) 15 June 1995, pages 2-7.	1, 2, 4 ----- 3, 5-34, 37-43

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